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Center of Excellence for Therapeutic Individualization for Breast Cancer
Annual Report: General Overview

The report submitted herein includes reports from subcontractors involved in the COE, which explain in detail the efforts of the COE during the past year. This report will highlight the overall progress made by the Center of Excellence.

1. Creation of Research Infrastructure: During the past 12 months, the COE has maintained and expanded the research infrastructure necessary for carrying out the proposed research.
    a. Intra-program communications: We continue to perform regular monthly teleconferences linking the principal investigators, and our face-to-face meetings (at the December San Antonio Breast Cancer Symposium and at the May 2006 American Society of Clinical Oncology Meetings.
    b. Clinical program development: We continue to identify clinical sites that will participate in our clinical trials and have taken steps to insure that drugs will be available at our planned foreign site in Lima, Peru. We have initiated accrual on our first clinical protocol (COE01, the master clinical protocol that represents the centerpiece of the program). Successful completion of COE01 is directly linked to the number of HOG sites opened. The DOD IRB approval of sites unfortunately continues to represent the major barrier to carrying out the research plan of the COE; we hope that DOD IRB approval of sites will occur with greater speed in the future. Our second and third trials (COE02 and COE03) are proceeding in development. COE02, which evaluates bevacizumab benefit, is awaiting approval by the DOD IRB. COE03, involving another novel agent, is currently in development and will be submitted to the DOD IRB in the near future.
    c. Clinical specimen processing: The COE has developed procedures specific to the processing and shipping of clinical specimens from clinical sites to the Pathology Core laboratory. The Pathology Core Laboratory has recently relocated from at the University of Oklahoma to the University of Colorado (with the transfer there of Dr. Ann Thor, core leader). The Pathology Core lab has successfully processed tissues obtained as part of COE 01. Procedures for transfer of tissue from ex-US sites have been successfully developed, and await the approval of these sites by the DOD IRB for useful incorporation into the COE01 trial.
    d. Research Core Laboratories: A principal focus of the COE's teleconference has involved the prioritization of clinical specimens for research evaluation. A prioritization process has been developed and is ready for use pending initiation of the clinical protocol. A laboratory procedure manual has been developed for use. The core research laboratories have developed standards for tissue processing, and (as outlined elsewhere) have hired personnel for specimen processing.

As genomic and proteomic technology change, the research core laboratories are in the process of developing novel approaches to investigation of crucial issues. As mentioned in the Leyland-Jones section of this report, we are actively investigating the performance of genomic analyses on paraffin-embedded tissues, which will significantly expand our ability to analyze patient samples, As outlined in the Chang/Baylor/Genomics section, significant progress has been made by the Baylor subcontractor in genomic analyses for docetaxel. This should prove useful in subsequent analyses.

2. Consumer Advocacy core: The Consumer Core has been heavily involved with all of the above, participating regularly in teleconferences, reviewing clinical protocols and procedure manuals, and helping create information packets for patients potentially interested in the protocol. Two highly successful Research Advocates Symposia have now been held, the first as an all-day meeting on Saturday, April 30th, 2005, and the second as a series of on-line teleconferences (the Advocate Lecture Series) conducted in March 2006 for breast cancer advocates.

Respectfully submitted,

George W. Sledge, Jr. MD

Baylor University subcontract report

Commander
U.S. Army Medical Research and Materiel Command
504 Scott Street
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Subject: Annual Report for Genomics Core

Introduction

Optimal systemic treatment after breast cancer is the most crucial factor in reducing mortality in women with breast cancer. Adjuvant chemotherapy and hormonal treatment both reduce the risk of death in breast cancer patients. However, while estrogen receptors status predicts for response to hormone treatments, there are no clinically useful predictive markers for chemotherapy responses. All eligible women are therefore treated in the same manner. Even denoval drug resistance will result in treatment failures in many breast cancer patients. Currently, there are no methods available to distinguish those patients who are likely to respond to specific chemotherapies, and given the accepted practice of prescribing adjuvant treatment to most parties, even if the average expected benefit is slow, the selection of appropriate patients represents a major advance in the clinical management of breast cancer today.

We therefore set out to identify gene expression patterns in breast cancer specimens that might predict response to taxenes. Chemotherapy allows for the sampling of the primary tumor for gene expression analysis and for direct assessment of response to chemotherapy by following changes in tumor size during the first few months of treatment. Hence, chemotherapy provides an idea platform to rapidly discover predictive markers of chemotherapy response.

In this present study, we hypothesize through high quantitation of gene expression, grade is possible to access thousands of genes simultaneously, and expression patterns in different breast cancers might correlate with and thereby predict response to treatment. The purpose of this study was to (1) demonstrate that sufficient RNA could be obtained from core biopsies to access gene expression, (2) to identify groups of genes that could be used to distinguish primary breast cancers to responsive or resistance to different chemotherapies and (3) to identify gene pathways that could be important in a mechanism of action of these agents.

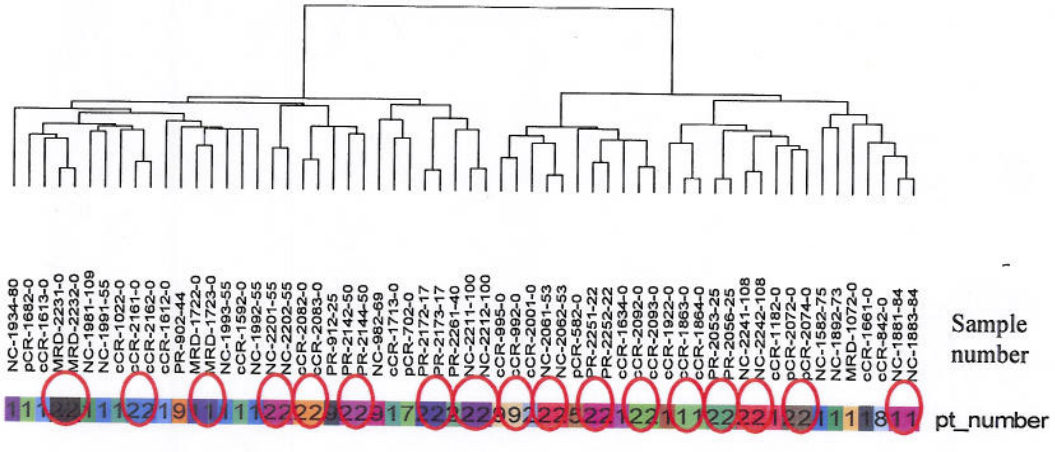
Body of Research

We have been improving gene expression arrays obtained from small tissue samples, as technical development. We measured each core biopsy obtained from primary breast cancers as approximately 1 cm x 1 mm. As these core biopsies were too small for micro dissection, we ascertained the tumor cellularity of the pretreatment core biopsies. In general, the core biopsies showed good tumor cellularity with median tumor cellularity of 75% (range 40-100%). Each core biopsy yielded 3-6 mg of total RNA, which is more than sufficient to generate approximately 20 mg of label cRNA needed for hybridization with the Affymetrix U133A Genechip, using the manufacturer's standard protocols. We have also experimented with laser microdissection of tumors of lower tumor cellularity.

We have begun to establish gene expression patterns for AC sensitivity and resistance. We examined forty patients with primary breast cancers greater than 3 cm were entered into a phase II neoadjuvant AC (Adriamycin 60 mg/m2, cyclophosphamide 600 mg/m2, 6 cycles, q-3weeks). Duplicate core biopsies were obtained in 20 patients, yielding a total of 60 core biopsies. Total RNA was isolated from individual core biopsies, and cRNA was labeled and

hybridized onto Affymetrix U133A Gene Chips. By unsupervised hierarchical clustering, gene expression from the same tumors tightly clustered together in 17/20 pairs (85%), indicating that inter-tumoral variation was greater than intra-tumoral heterogeneity or technical differences (Figure 1). Of 233 differentially expressed genes, pathways up-regulated in sensitive tumors included oncogenes (AREG, EGFR), cytokines and other inflammatory genes, stress-related proteins (interleukins, interferons), metabolism ma(lipoprotein lipases), and estrogen-related pathways (ER, IRS1). Resistant tumors expressed genes promoting differentiation (TFF3, FGF), invasion (MMP1), and anti-proliferation genes (CDKN1B). This molecular portrait for AC differs from our published profile for docetaxel chemoresistance (1). Gene expression arrays offers a potential predictive test of sensitivity for different treatments, and may thereby allow selection of the most appropriate therapy for breast cancer patients, as we propose here.

Figure 1. Unsupervised clustering of 60 samples from 40 patients. Most of the duplicate pairs, as shown by the red circles, from the same tumor (17/20 pairs, 85%) tightly clustered together, indicating that inter-tumoral variation was greater than tumor heterogeneity or technical variability.



References:

1. Chang, J. C., Wooten, E. C., Tsimelzon, A., Hilsenbeck, S. G., Guiterrez, M. C., Elledge, R. M., Mohsin, S., Osborne, C. K., Chamness, G. C., Allred, D. C., and O'Connell, P. Gene expression profiling predicts therapeutic response to docetaxel (Taxotere(tm)) in breast cancer patients. Lancet, *362:* 280-287, 2003.

Hoosier Oncology Group subcontract report

McGill University subcontract report

Introduction

The primary objective of the pharmacodynamics/pharmacogenomics core facility is to develop user-friendly techniques readily available to the clinician for measuring a specific aspect of response and/or toxicity, which will lead to the individualization of therapy. Critical determinants that govern individual responsiveness will be identified. These include markers and kinetic rate or metabolic outcome, which are often referred to as a pharmacokinetic or pharmacodynamic "signature". One critical advantage of measuring these "signatures" is that they will be directly compared to and contrasted with the genomic and proteomic analyses.

In order to identify such pharmacokinetic signatures, a number of techniques have been established in our lab that include: 1) FISH (Fluorescent *In Situ* Hybridization) used for the detection of amplification or deletion of the topoisomerase II A gene (TOP2A), a well known target of anthracyclines; 2) ELISA assays to evaluate tumoral protein levels of ALDH1; 3) qRT-PCR (quantitative reverse transcriptase-polymerase chain reaction) to measure expression levels of key enzymes involved in the metabolism of capecitibine. In the case of Vinorelbine (Arm C), we have established RT-PCR protocols enabling us to measure mRNA levels of two promising biomarkers, namely β-tubulin III and stathmin from fresh frozen tissue. RT-PCR will also be used to measure tumor expression levels of deoxycytidine kinase (dCK) and ribonucleotide reductase M1 (RRM1), two enzymes involved in the metabolic pathway of gemcitabine. 4) IHC (immunohistochemical) assays, set up in collaboration with Dr. MacKey, will be used to assess levels of two transporters, the human concentrative nucleoside transporter (hCNT) and the human equilibrative nucleoside transporter 1 (hENT1), both involved in the cellular transport of gemcitabine (Arm D).

Correlation of the protein/enzyme activity profile with disease state, therapy and drug response will provide invaluable insight into monitoring inter-individual variations in efficacy and toxicity. Moreover, these observations could be used to help select appropriate drug and dosage regimens for each patient.

Protocol development:

Arm A: Doxorubicin and Cyclophosphamide

Doxorubicin

The enzyme Topoisomerase II A, which catalyses the breakage and reunion of double-stranded DNA, plays an important role in a number of fundamental nuclear processes including DNA transcription, replication and recombination. In addition, topoisomerases are required for maintaining proper chromosome structure and segregation[1]. Type II topoisomerases are targets for the anthracycline class of anticancer drugs, such as doxorubicin and epirubicin, which are also termed topoisomerase inhibitors[2].

The TOP2A FISH assay is used to determine the copy number of the TOP2A gene, which is located on chromosome 17q21-q22, using the chromosome 17 centromere region (Cen17) as a reference[3]. Two copies of the TOP2A gene are present in all normal diploid cells and in some carcinomas (figure 1), whereas, the TOP2A/Cen17 ratio is higher in cells when the TOP2A gene is amplified (usually defined as >2) (figure 2) or lower in cells when the TOP2A gene is deleted (usually <1).

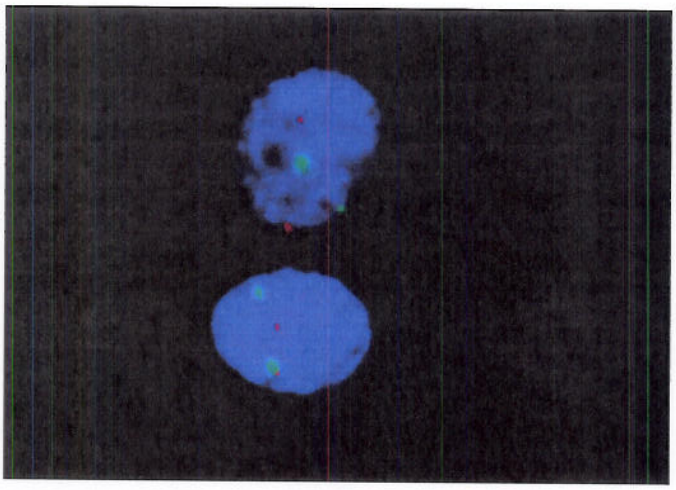


Figure 1: Breast carcinoma with two fluorescent signals for chromosome 17 centromere region (green) and two signals for TOP2A (red) per nucleus.

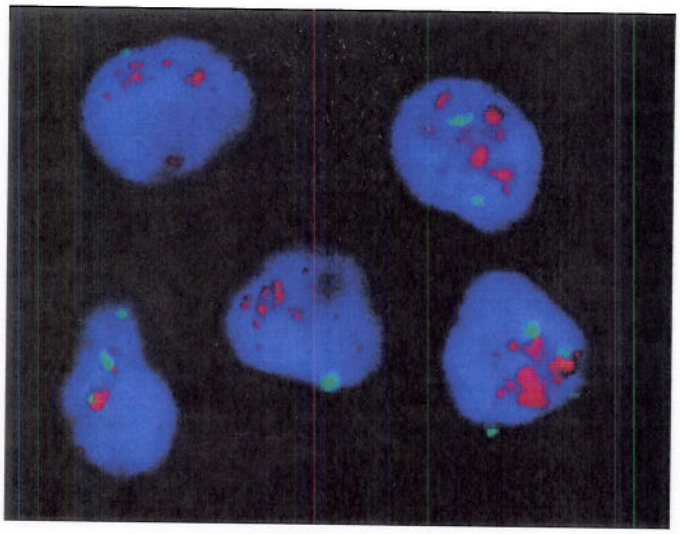


Figure 2: Breast carcinoma with two fluorescent signals for chromosome 17 centromere region (green) and >10 signals for TOP2A (red) per nucleus.

Cyclophosphamide

In preclinical models, molecular determinants of cellular sensitivity to the oxazaphosphorine cyclophosphamide (CPA) include two aldehyde dehydrogenases, namely, ALDH1A1 and ALDH3A1[4]. Specifically, cellular sensitivity to oxazaphophorines is inversely related to the cellular content of these enzymes because they each catalyze the detoxification of these agents[5]. Quantification of ALDH1A1 is performed using an indirect enzyme-linked immunoadsorbant assay (ELISA)[6,7]. Briefly, enhanced protein binding 96 well plates (Corning Inc) were coated with a goat anti-ALDH1 antibody (Abcam), tissue homogenates were added and incubated for 2h. After washing, a horseradish peroxidase (HRP) conjugated anti-goat antibody was added followed by the HRP substrate and was read using a plate reader.

Diltiazem

CPA is a DNA-alkylating agent commonly used in cancer chemotherapy. This anticancer agent is administered as a prodrug that is activated by a liver cytochrome P450-catalysed 4-hydroxylation reaction that yields the therapeutically active DNA cross-linking phosphoramide mustard and the byproduct acrolein[8]. In patients with cancer, CPA is primarily activated in the liver, a tissue rich in P450 activity, followed by transport of the activated metabolites to the tumor via blood flow. Metabolic phenotype can be determined by measuring the ratio of diltiazem, a non cytotoxic probe drug, and its major metabolites in timed plasma and urine samples.

There are a number of methodologies to measure diltiazem and its major metabolites in plasma using HPLC, however, we decided to use the method published by Yeung *et al*[9] with minor modifications. Our HPLC system consists of a PerkinElmer TCCS QUAT diode array system (Sheldon, CT, USA), a PerkinElmer series 200 fluorescence detector, a PerkinElmer series 200 autosampler, and a series 200 Peltier oven. The stationary phase is composed of an ODS, Spheri-5, C18, 5 µm particle size, 250 x 4.6 mm i.d. Brownlee Column. The mobile phase is composed of a mixture of methanol, 0.04 M ammonium acetate, and acetonitrile (40:36:24) and 0.04% triethylamine, with pH adjusted to 7.3 using glacial acetic acid. The HPLC system is operated isocratically at 30°C with a flow rate of 1.2 mL/min. The detector wavelength is set at 237 nm. Diltiazem hydrochloride (Sigma) and the major diltiazem metabolites, which include deacetyl diltiazem (M1), *N*-monodemethyl diltiazem (MA), deacetyl *N*-monodemethyl diltiazem (M2), and deacetyl O-monodemethyl diltiazem (M4), were the kind gift of Tanabe Seiyaku Co. Ltd. (Japan). Stock solutions of Diltiazem (1mg/ml) and the internal standards, verapamil (1mg/ml) and imipramine (1mg/ml), are prepared by dissolving the appropriate amount of drug in methanol and stored at -20°C.

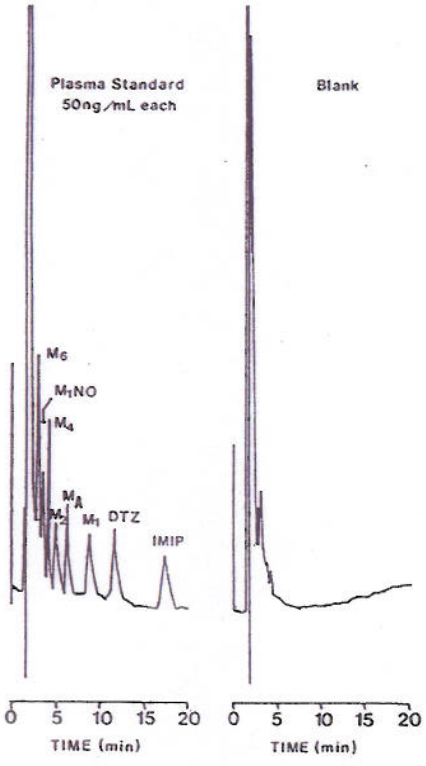


Figure 3: Typical HPLC chromatogram of diltiazem and its major metabolites.

Arm B: Capecitabine

Fluoropyrimidines, with 5-fluorouracil (5-FU) as one of its principal representatives of this class of chemotherapeutic agents, have been the standard treatment for a wide range of common solid tumors including breast cancer. Attempts to increase the efficacy and tolerability of fluoropyrimidine treatment have led to the development of capecitibine, a fluoropyrimidine prodrug, which is preferentially activated at the tumor site by the enzyme thymidine phosphorylase (TP)[10]. Capecitabine and its intermediate metabolites, 5'deoxy-5-fluorocytidine (5'-DFCR) and 5'-deoxy-5-fluorouridine (5'DFUR), are not cytotoxic but become effective only after they have been converted to 5-FU by TP[11]. One of the principal mechanisms of 5-FU action is inhibition of the enzyme thymidilate synthase (TS) by fluorodeoxyuridine monophosphate (FdUMP), one of several 5-FU metabolites. TS is an important enzyme in pyrimidine metabolism which is crucial for *de novo* synthesis of thymidine nucleotides. Another enzyme, that most likely plays a crucial role in the antitumor activity of 5-FU is dehydropyrimidine dehydrogenase (DPD), because DPD is responsible for the catabolic conversion of 5-FU to an inactive metabolite and decreases 5-FU levels within cells[12,13].
Therefore, the fluoropyrimidine pathway enzymes, TP, TS and DPD, may be potential candidate biomarkers that could be used to predict tumor response to capecitabine.

Quantitative assessment of gene expression by RT-PCR assays enables the use of limited samples of fresh frozen tissue as well as of formalin fixed paraffin-embedded (FFPE) tissue in a very short time. Quantitative analysis of DPD, TP or TS mRNA levels is conducted by a two-step procedure. In the first step, cDNA is reverse transcribed from total RNA using AMV reverse transcriptase and random hexamer priming. In the second step, a fragment of DPD, TP or TS-encoding mRNA is amplified from the cDNA by PCR using gene specific primers. The amplicon is detected by fluorescence using a specific pair of hybridization probes[14]. In the same cDNA preparation but in a separate PCR reaction, mRNA encoding for glucose-6-phosphate dehydrogenase (G6PDH) is quantified for use as a reference (housekeeping) gene. The DPD, TP or TS expression level is quantified relative to G6PDH and a calibrator RNA, therefore, a standard curve is not required for its determination (figures 4 to 6).

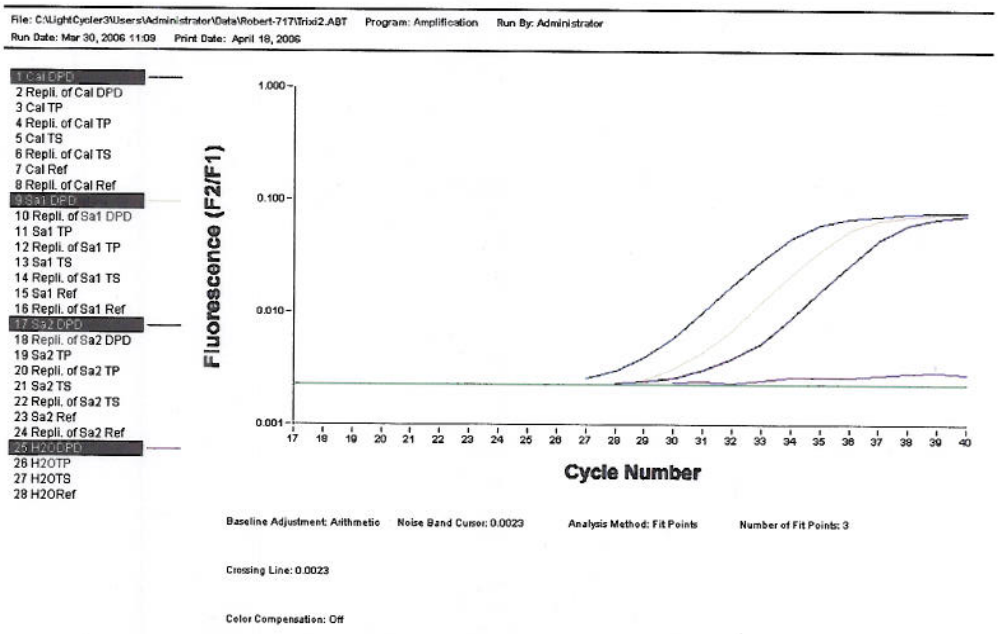


Figure 4: Representative RT-PCR for DPD using mRNA extracted from a single 10 µm formalin fixed paraffin embedded tissue slice. From left to right; calibrator, sample #1, sample #2, -RNA.

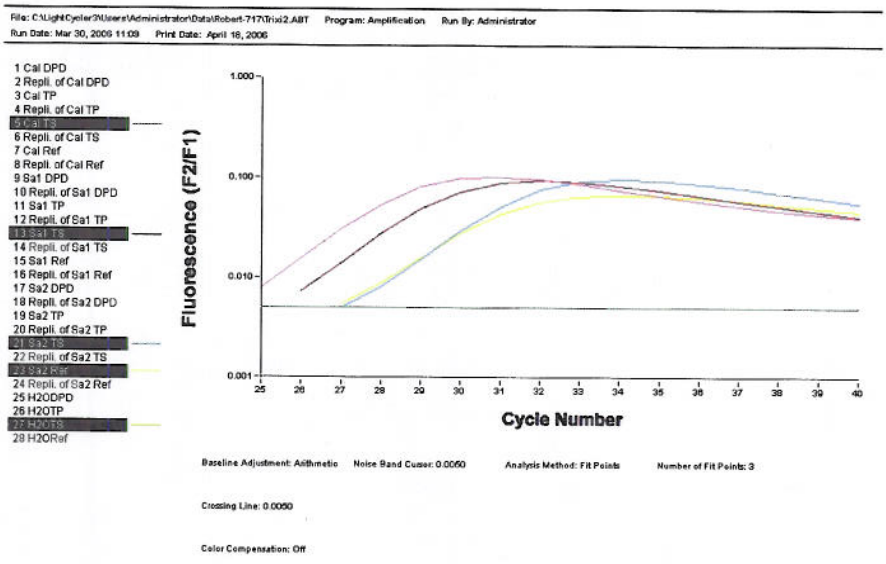


Figure 5: Representative RT-PCR for TS using mRNA extracted from a single 10 µm formalin fixed paraffin embedded tissue section. From left to right; calibrator, sample #1, sample #2, -RNA (no amplified product detected).

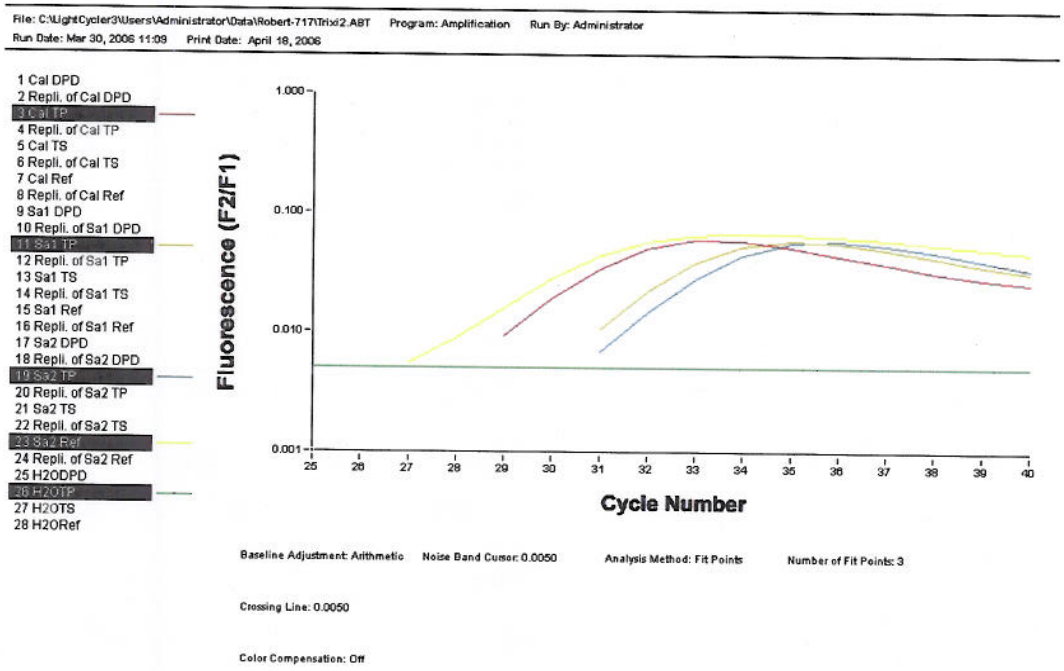


Figure 6: Representative RT-PCR for TP using mRNA extracted from a single 10 µm formalin fixed paraffin embedded tissue section. From left to right; reference, calibrator, sample #1, sample #2, -RNA (no amplified product detected).

Arm C: Vinorelbine

Antitubulin agents such as taxanes and vinorelbine are widely used in the treatment of patients with breast cancer. The target of these compounds, microtubules, are complex polymers consisting of α/β tubulin heterodimers and a variety of microtubule-associated proteins to which these compounds bind. The expression of certain tubulin isotypes have often been found to be correlated with their sensitivity to antitubulin agents[15]. Recently, Rosell *et al* used quantitative RT-PCR to analyze the expression of β-tubulin III and stathmin mRNA isolated from FFPE tumor biopsies from NSCLC patients treated with vinorelbine and showed that time to progression was influenced by β-tubulin III and stathmin levels[16].

Quantitative assessment of β-tubulin III and stathmin expression is conducted in a similar manner described in Arm B except that no calibrator RNA is used and quantification is done using a standard curve (figure 7).

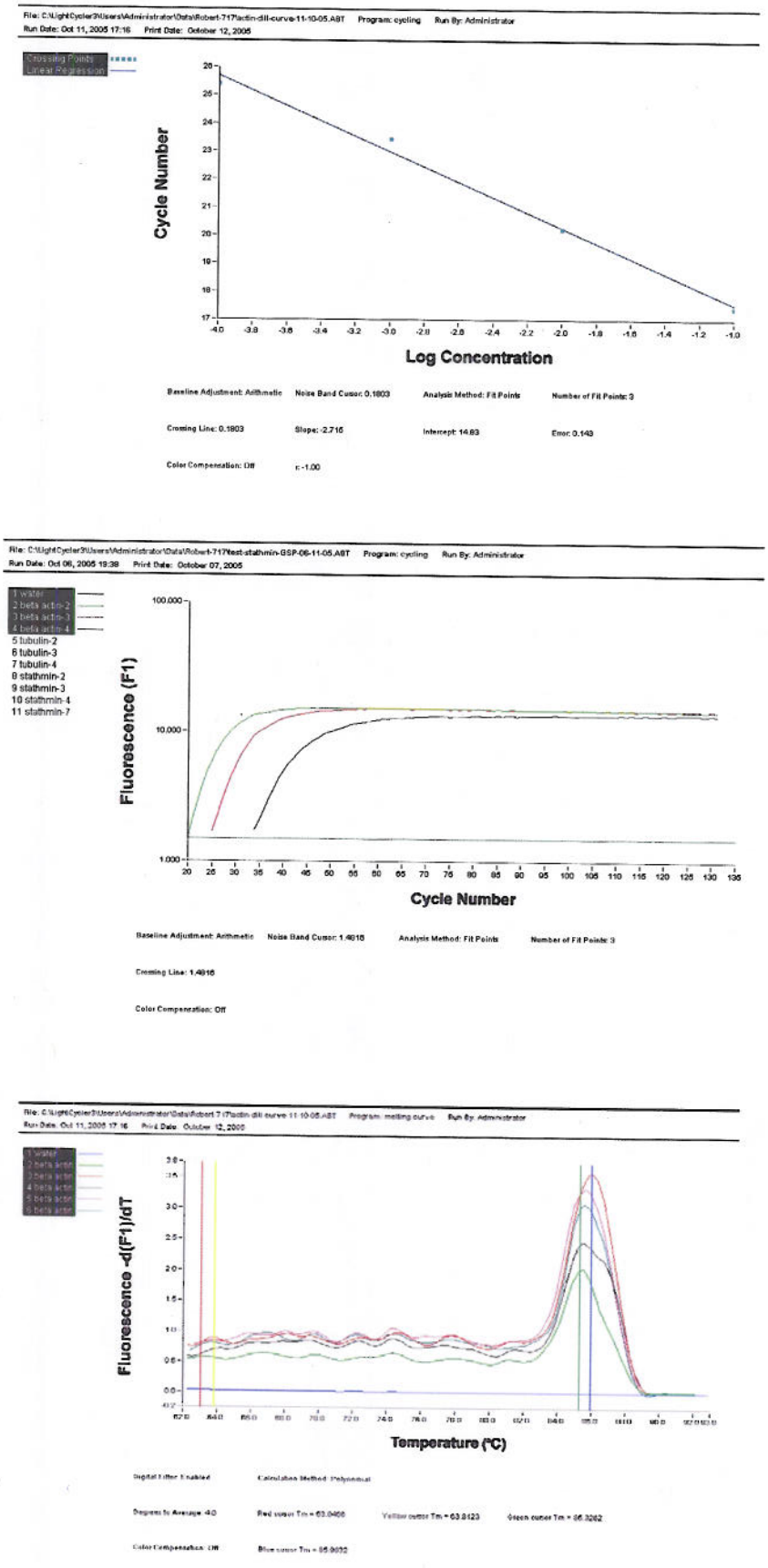


Figure 7: Representative RT-PCR analysis of actin mRNA expression in fresh frozen tissue. Standard curve (top), amplification (middle) and melting curve analysis (bottom).

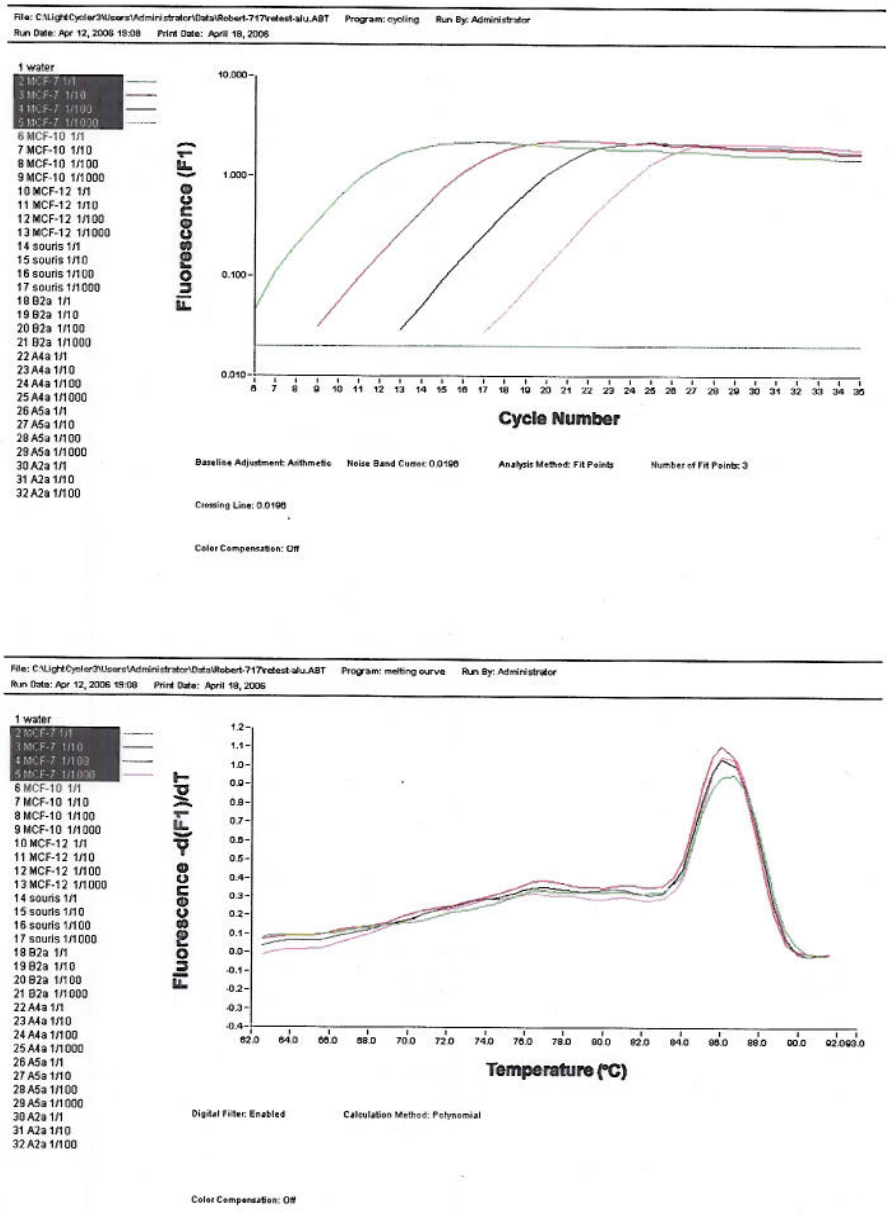


Figure 8: Representative RT-PCR analysis of β-tubulin mRNA expression in fresh frozen tissue. Amplification (middle) and melting curve analysis (bottom).

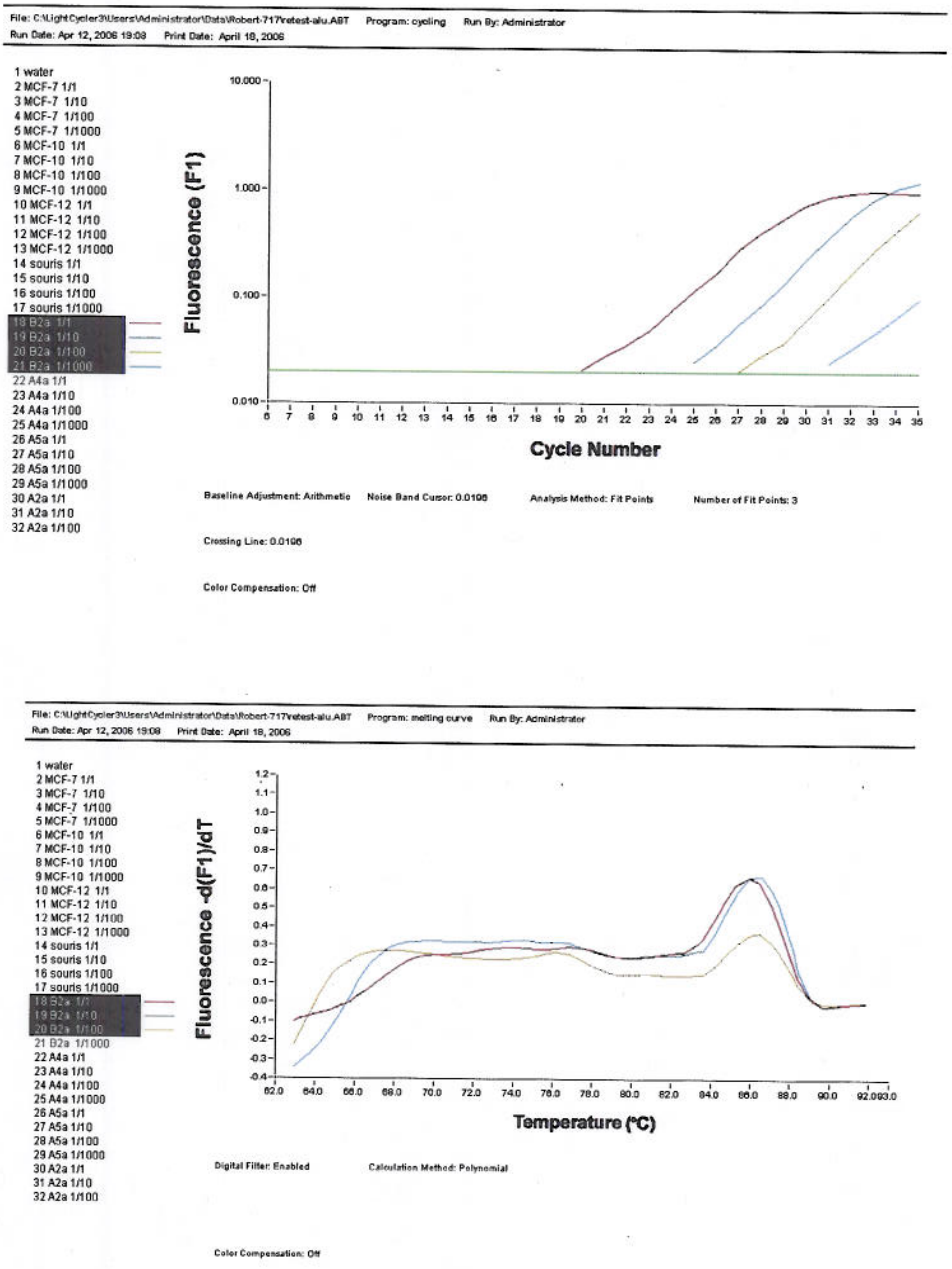


Figure 9: Representative RT-PCR analysis of stathmin mRNA expression in fresh frozen tissue. Amplification (middle) and melting curve analysis (bottom).

Arm D: Gemcitabine

Activation of gemcitabine requires phosphorylation to mono-, di-, and triphosphates. Similar to the structurally and functionally related deoxycytidine analog ara-C, the first crucial step is phosphorylation catalyzed by deoxycytidine kinase (dCK)[17]. However, in contrast to ara-C, gemcitabine has multiple intracellular targets; up- or down-regulation of these targets may confer resistance to this drug. Recent studies show a strong correlation between sensitivity to gemcitabine as well as the most important

metabolite of gemcitabine, dFdCTP and dCK activity. A potential important role could also be played by ribonucleotide reductase (RRM1), one of gemcitabine's targets, along with members of specialized transport systems required for the passage of nucleoside analogs. dCK and RRM1 may be potential candidate biomarkers that could be used to predict tumor response to gemcitabine.

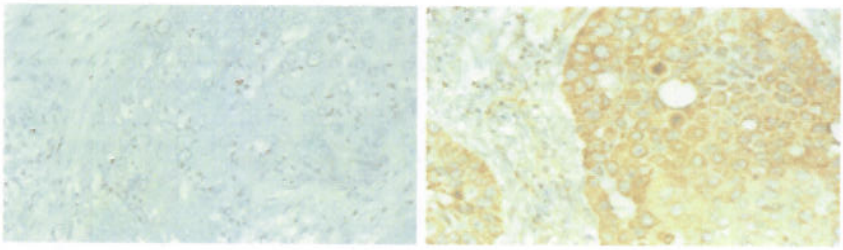


Figure 10: Immunocytochemical staining of deoxycytidine kinase (dCK). Phosphate buffered saline negative control (left) and sample with high dCK expression (right).

DASL assay – Proof of concept studies

In order to get the DASL assay up and running at McGill University & Genome Quebec Innovation Centre (MUGQIC), DASL assay software was provided by Illumina so as to allow the conversion of the BeadStation to one capable of running the DASL assay Sentrix® Universal Array platform. Analysis software from Illumina was also installed. To ensure that the DASL assay platform was functioning properly, two initial DASL assay experiments have to date have been performed, the first one with technicians from Illumina present, and are described below.

DASL Assay Experiment #1

Total RNA was prepared from FFPE breast tumor tissue from two patients provided to us by Dr. Charlie Catzavelos, St. Mary's Hospital, Montreal, QC.

- Patient Sample 1
  - 13006: Intermediate grade, invasive ductal carcinoma with lymphatic invasion. LN+ve.
  - ER - Strong positive
  - PR - Moderate
  - HER2 - Positive by FISH (fluorescence in situ hybridization)
- Patient Sample 2
  - 6875: High grade, invasive ductal carcinoma with profound lymphatic invasion. LN+ve.
  - ER - Negative
  - PR - Negative
  - HER2 - Positive by FISH

Nine x 5 µm sections from each patient's FFPE tumor block were used to extract total RNA from using the High Pure RNA Paraffin Kit and following the manufacturer's instructions. RNA was analyzed on the Agilent Bioanalyzer 2100 and the tracings are shown in Fig 1. The majority of RNA fragments were greater than 100 bp, with a small fraction being greater than 700 bp. The total RNA yield for the two samples was 1.74 µg and 3.40 µg, respectively. The RNA samples were split into two aliquots and each subjected to DASL assays in duplicate, for a total of 8 DASL assays, using the 502-gene human cancer panel from Illumina (gene list available at www.illumina.com). Representative Intensity plots are shown comparing sample 1 with 1a, sample 2 with 2a and then sample 1 with sample 2 (Fig. 11).

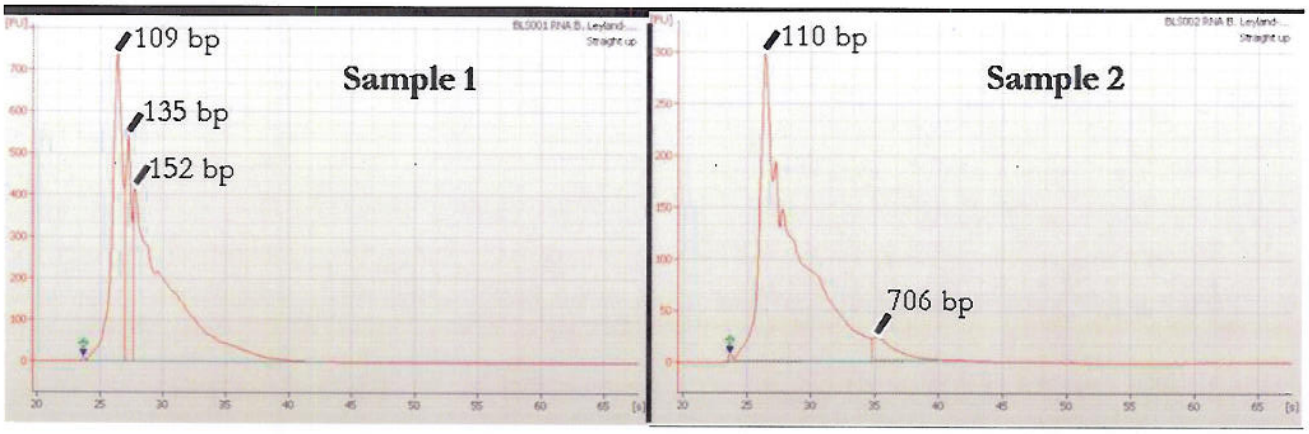


Fig. 11. Tracings are shown for total RNA from the two samples subjected to analysis on the Agilent Bioanalyzer 2100.

Duplicate samples show similar signal intensities, with differences occurring mainly in the lower intensity range. Greater differences in intensity, from low to high, are evident when comparing sample 1 with sample 2. Importantly, the expression data from the DASL assay for the receptor genes correlates with the immunohistochemical (IHC) and FISH data for each patient's sample as shown in Fig. 12.

HER2 is well expressed in both samples and TFF1, an estrogen inducible gene, shows increased expression in the ER-positive sample 1. A heat map for a number of genes that play an important role in cancer biology is shown in Fig. 14A. The DASL assay can readily pick out differences in gene expression between these two breast tumor samples as well as in the four additional breast tumor samples described below.

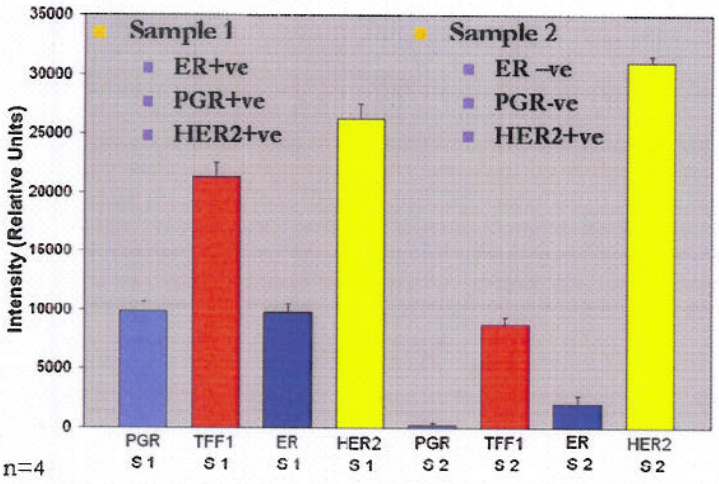


Fig. 12. The average intensity of receptor genes for samples 1 and 2 in the DASL assay. TFF1 (Trefoil Factor 1) is an ER-inducible gene. ER, estrogen receptor; PGR, progesterone receptor.

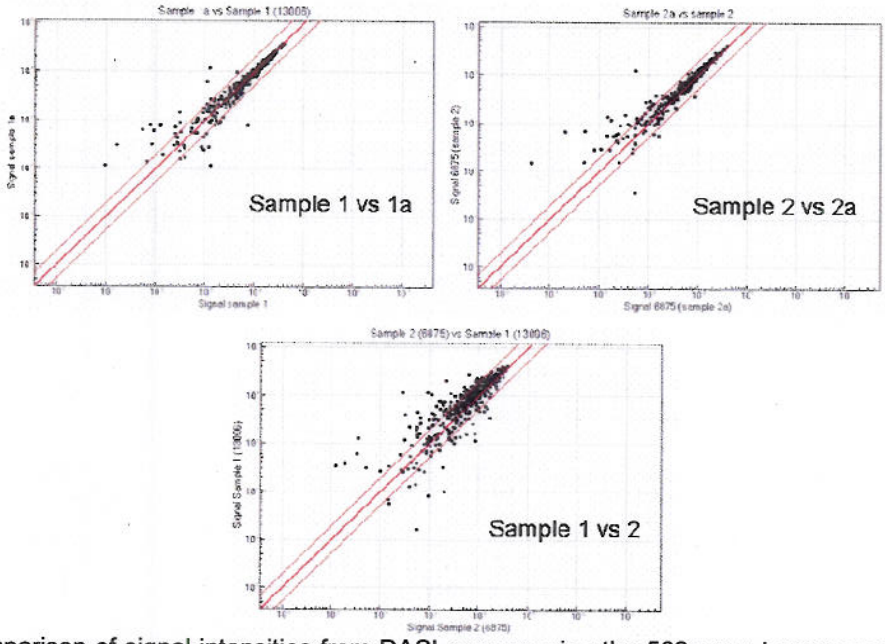


Fig. 2. Comparison of signal intensities from DASL arrays using the 502-gene human cancer panel for samples 1 versus 1a, 2 versus 2a and sample 1 versus 2.

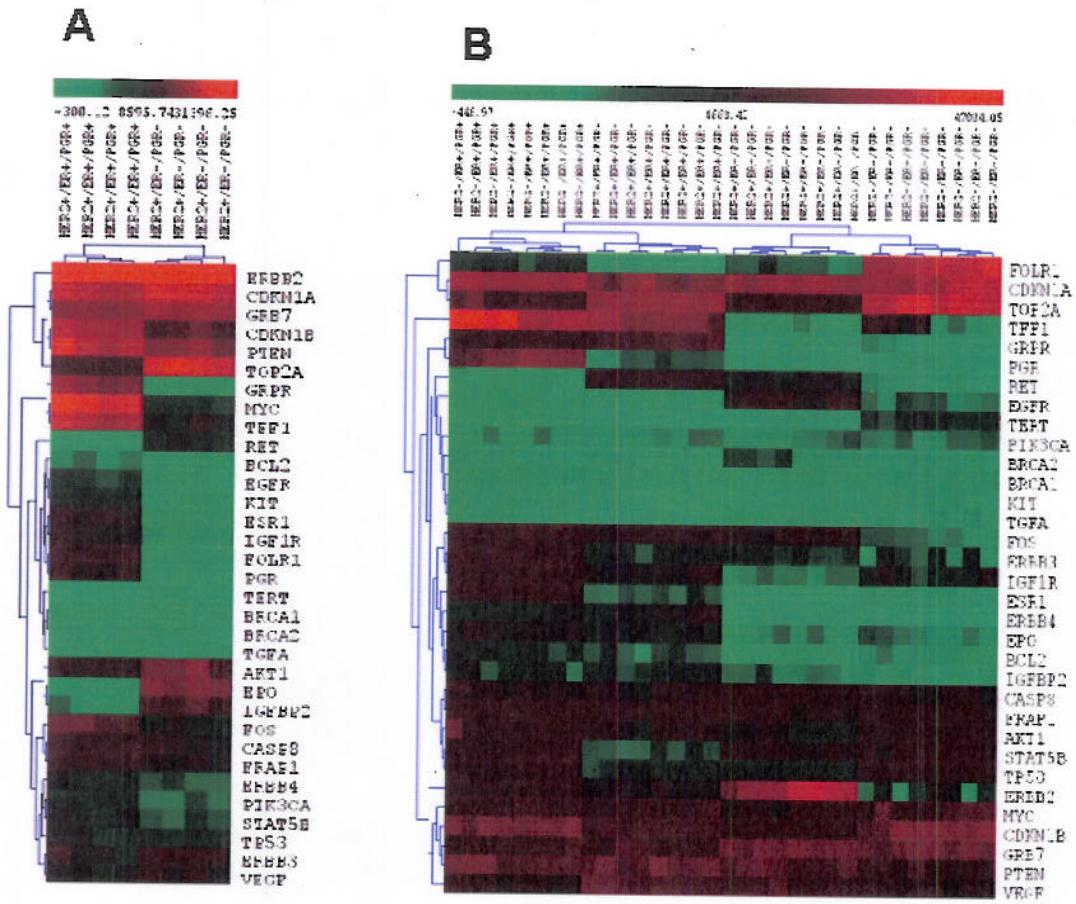


Fig. 14. Hierarchical clustering (A) of two patient's tumor samples extracted in duplicate and hybridized in duplicate on one Sentrix® Array Matrix from Illumina and (B) of four patient's tumor samples extracted in quadruplicate and hybridized in duplicate on two Sentrix® BeadChip arrays from Illumina.

DASL Assay Experiment #2

Total RNA was prepared from FFPE breast tumor tissue from four patients. The patients were selected based on their receptor profile

- Patient Sample 1
  - 5564: High grade, invasive ductal carcinoma with lymphatic invasion. LN+ve.
  - ER - Strong positive
  - PR - Negative
  - HER2 - Positive by IHC
- Patient Sample 2
  - 6786: High grade, invasive ductal carcinoma with lymphatic invasion. LN+ve.
  - ER - Negative
  - PR - Negative
  - HER2 - Negative
- Patient Sample 3
  - 3524: High grade, invasive ductal carcinoma with lymphatic invasion. LN+ve.
  - ER - Negative
  - PR - Negative
  - HER2 - Positive by IHC
- Patient Sample 4
  - 9152: High grade, invasive ductal carcinoma with no lymphatic invasion. LN-ve.
  - ER - Positive
  - PR - Positive
  - HER2 - Negative (by FISH)

Quadruplicate total RNA extractions were performed on FFPE tumor samples using three x 5 µm sections for each in conjunction with the High Pure RNA Paraffin Kit (Roche) and following the manufacturer's instructions. The RNA yields for the 16 RNA preps are shown in Table 1.

Table 1. RNA yields for quadruplicate preparations from 4 breast cancer patients.

Tumor Sample #	RNA Conc. (ng/µL)	Yield (µg)
SO-05-5564	209	8.4
SO-05-5564	164	6.6
SO-05-5564	236	9.4
SO-05-5564	295	11.8
SO-05-6786	166	6.6
SO-05-6786	145	5.8
SO-05-6786	143	5.7
SO-05-6786	134	5.4
SO-05-3524	83	3.3
SO-05-3524	132	5.3
SO-05-3524	95	3.8
SO-05-3524	78	3.1
SO-05-9152	93	3.7
SO-05-9152	68	2.7
SO-05-9152	116	4.6
SO-05-9152	90	3.6

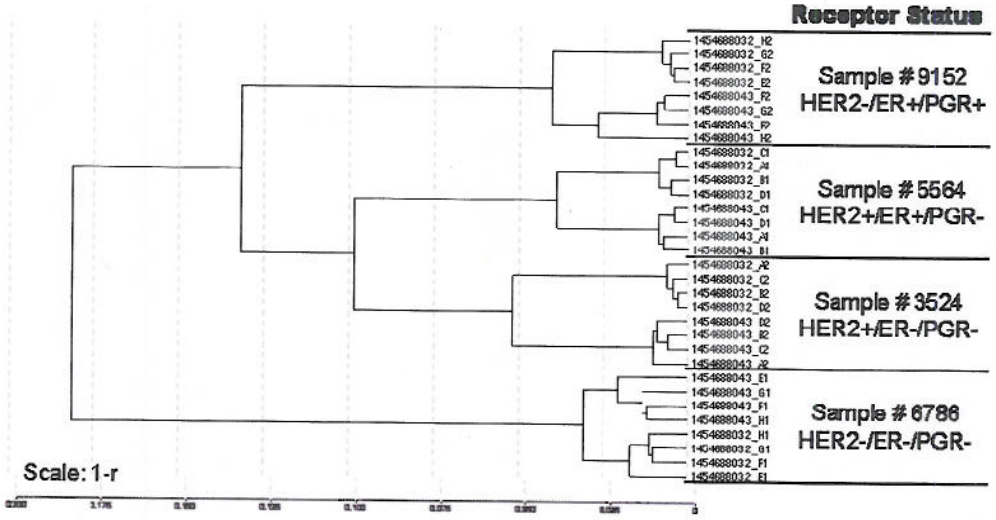


Fig.15. Dendogram of FFPE breast tumor samples from Exp. #2 analyzed using the DASL assay.

The RNA yields for the quadruplicate samples were excellent and would be enough to support several DASL assay as well as qRT-PCR experiments. The quality of the

RNA was assessed by both the Agilent Bioanalyzer 2100 and to qRT-PCR of the RPL13a (ribosomal protein L13a). Bioanalyzer tracings showed slightly less degradation then occurred with the previous two samples and RPL13a was readily amplified from all samples, although Ct values (range 29-35) were at the high end of the QC range. The DASL assay was performed in duplicate and assay results were similar for the quadruplicate samples as shown in the dendogram in figure 15. The dendogram shows that within each quadruplicate group of samples (each done in duplicate DASL assays) reproducibility was very good. The sample (6786) that was triple negative for receptor expression was least similar to the other three samples, as what one would expect from previously published microarray experiments, with the HER2-positive samples (5564 and 3524) being most similar to each other. Expression of selected genes, important in cancer biology, is shown as a heat map in Fig. 14B. The intensity data for the receptor genes (ER, PGR, HER2) and TFF1 is shown in figure 16. For the most part the DASL assay data correlates with the IHC and FISH data for the 4 samples. The most obvious discord is with HER2 expression in patient's sample 5564; positive by IHC but relatively weakly expressed in the DASL assay. The sample has been sent for FISH analysis to confirm the IHC result. The estrogen inducible gene, TFF1, appears to track with ER levels; increased ER intensity results in increased TFF1 intensity.

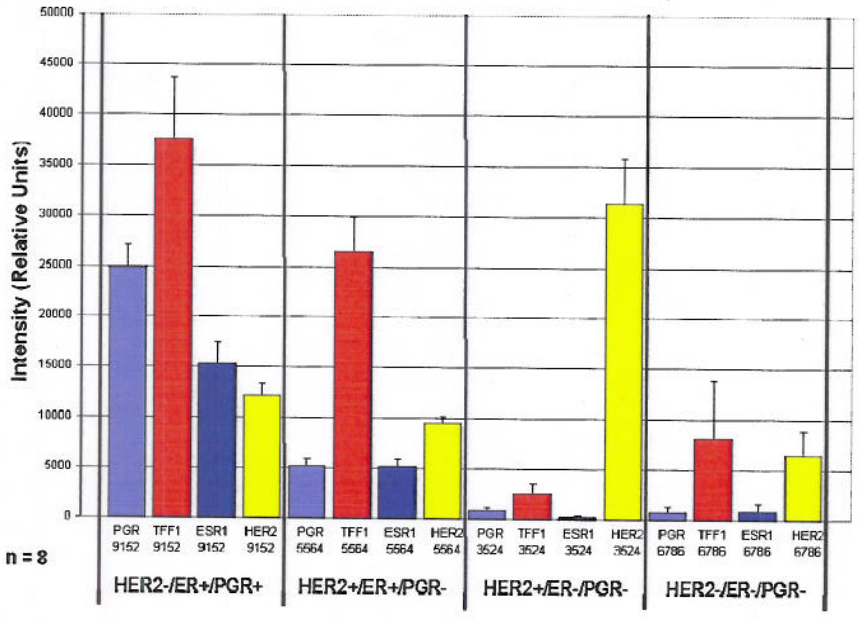


Fig. 16. The average intensity of receptor genes for samples from Exp. #2 in the DASL assay. TFF1 (Trefoil Factor 1) is an estrogen inducible gene. ER, estrogen receptor; PGR, progesterone receptor.

The preliminary data shows that 1) more than enough total RNA can be extracted from 3 x 5 µm sections from FFPE breast tumor tissue blocks, 2) poor quality, degraded RNA is suitable for use in the DASL assay as long as RNA fragments are greater than 50 bp in size, 3) the assay showed good reproducibility, 4) the DASL assay data for receptor expression correlated with IHC and FISH data, and 4) MUGQIC has the capability and appropriate instrumentation to perform the DASL assay.

References

1. Wang,J.C. Cellular roles of DNA topoisomerases: a molecular perspective. *Nat. Rev. Mol. Cell Biol.* **3**, 430-440 (2002).

2. Hande,K.R. Topoisomerase II inhibitors. *Cancer Chemother. Biol. Response Modif.* **21**, 103-125 (2003).

3. Jarvinen,T.A., Tanner,M., Barlund,M., Borg,A. & Isola,J. Characterization of topoisomerase II alpha gene amplification and deletion in breast cancer. *Genes Chromosomes. Cancer* **26**, 142-150 (1999).

4. Sladek,N.E., Kollander,R., Sreerama,L. & Kiang,D.T. Cellular levels of aldehyde dehydrogenases (ALDH1A1 and ALDH3A1) as predictors of therapeutic responses to cyclophosphamide-based chemotherapy of breast cancer: a retrospective study. Rational individualization of oxazaphosphorine-based cancer chemotherapeutic regimens. *Cancer Chemother. Pharmacol.* **49**, 309-321 (2002).

5. Sladek,N.E. Aldehyde dehydrogenase-mediated cellular relative insensitivity to the oxazaphosphorines. *Curr. Pharm. Des* **5**, 607-625 (1999).

6. Sreerama,L. & Sladek,N.E. Primary breast tumor levels of suspected molecular determinants of cellular sensitivity to cyclophosphamide, ifosfamide, and certain other anticancer agents as predictors of paired metastatic tumor levels of these determinants. Rational individualization of cancer chemotherapeutic regimens. *Cancer Chemother. Pharmacol.* **47**, 255-262 (2001).

7. Sreerama,L. & Sladek,N.E. Cellular levels of class 1 and class 3 aldehyde dehydrogenases and certain other drug-metabolizing enzymes in human breast malignancies. *Clin. Cancer Res.* **3**, 1901-1914 (1997).

8. Colvin,M., Padgett,C.A. & Fenselau,C. A biologically active metabolite of cyclophosphamide. *Cancer Res.* **33**, 915-918 (1973).

9. Yeung,P.K., Montague,T.J., Tsui,B. & McGregor,C. High-performance liquid chromatographic assay of diltiazem and six of its metabolites in plasma: application to a pharmacokinetic study in healthy volunteers. *J. Pharm. Sci.* **78**, 592-597 (1989).

10. Miwa,M. *et al.* Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5-fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue. *Eur. J. Cancer* **34**, 1274-1281 (1998).

11. Miwa,M. *et al.* Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5-fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue. *Eur. J. Cancer* **34**, 1274-1281 (1998).

12. Lu,Z., Zhang,R. & Diasio,R.B. Dihydropyrimidine dehydrogenase activity in human peripheral blood mononuclear cells and liver: population characteristics, newly identified deficient patients, and clinical implication in 5-fluorouracil chemotherapy. *Cancer Res.* **53**, 5433-5438 (1993).

13. Johnson,M.R., Wang,K., Tillmanns,S., Albin,N. & Diasio,R.B. Structural organization of the human dihydropyrimidine dehydrogenase gene. *Cancer Res.* **57**, 1660-1663 (1997).

14. Wittwer,C.T., Herrmann,M.G., Moss,A.A. & Rasmussen,R.P. Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* **22**, 130-138 (1997).

15. Nicoletti,M.I. *et al.* Expression of beta-tubulin isotypes in human ovarian carcinoma xenografts and in a sub-panel of human cancer cell lines from the NCI-Anticancer Drug Screen: correlation with sensitivity to microtubule active agents. *Clin. Cancer Res.* **7**, 2912-2922 (2001).

16. Rosell,R. *et al.* Transcripts in pretreatment biopsies from a three-arm randomized trial in metastatic non-small-cell lung cancer. *Oncogene* **22**, 3548-3553 (2003).

17. Bergman,A.M., Pinedo,H.M. & Peters,G.J. Determinants of resistance to 2',2'-difluorodeoxycytidine (gemcitabine). *Drug Resist. Updat.* **5**, 19-33 (2002).

University of Oklahoma subcontract report

Pathology Core

During the second year of this grant we have received specimens from two separate patients enrolled in protocol COE01. All specimens from the first patient were processed for histology since neither the originally submitted specimen, nor the specimen submitted for clinical pathologic interpretation at the collaborating institution contained any tumor tissue. We subsequently received the final blood specimens from the first patient at the completion of treatment. In addition, one of the investigators flew to Indianapolis in September 2005, before the first case was accrued to be trained in the web based specimen accrual and log in system. Both the Core Director and co-investigator met with all other investigators and the project director at the ASCO meeting in May 2005. In addition the co-investigator also met with all other investigators and the project director at the San Antonio Breast Cancer Symposium in December 2005.

The core laboratory re-located to the University of Colorado Health Sciences Center at Fitzsimons in Aurora Colorado in April 2006. No problems have occurred during the transition. Once the DOD IRB office approves the additional sites we anticipate that the accrual rate will rise.

Research Advocacy Network subcontract report

Introduction

Advocate participation in this research study allows them to better understand the science, the methodology and the results so they can take the lead in informing the advocacy community about this important project. The patient advocates create and maintain a network of advocates and advocate organizations.

The Patient Advocates work closely with the Clinical Trial Core to develop patient education materials and expand recruitment strategies. Plans are for: a brochure to provide general information to patients and a one-page summary explaining each study. All will be in English and Spanish.

Educational programs for advocates increase their awareness and understanding of the objectives of the Center. One-day programs offered at selected BCE sites will include researchers discussing their ongoing research, an overview of the Center and how advocates can be involved. Web conferences will offer expanded access for advocates nation-wide. Web site content will increase access and provide resources for patients and advocates.

During this reporting period, advocates in the Advocate Core participated with researchers to provide the patient perspective to all discussions. Face-to-face meetings enabled advocates to meet and interact with the PI and other researchers. Meetings covered topics such as expectations of the advocates and researchers; progress to date; and arrangements for the Advocate Lecture Series.

Advocates worked closely with the co-PIs of the study *Predicting Response and Toxicity in Patients Receiving Chemotherapy for Breast Cancer: A Multicenter Genomic, Proteomic, and Pharmacogenomic Correlative Study* to develop a one-page summary. This summary contained the title, the sponsor, the rational, the purpose, the design and the eligibility requirements of the study. The summary received local IRB approval.

Advocates and Center staff developed content for the Center's web site. The content was tailored to patients, the public and advocates and provides general information about the Center Of Excellence For Individualization Of Therapy In Breast Cancer. It provides resources educating the audience about the science of "omics" so integral to the Center's work. It also links to resources on tissue research and its importance to developing new therapies.

The Advocate Lecture Series was conducted on three Thursdays in March. Each lecture lasted one hour with a presentation by one of the Center's researchers and time for questions and answers. Sixty – 70 advocates registered for each lecture. The goal was to inform advocates about 1) the IU/DOD Breast Cancer Centers of Excellence Grant activities 2) the science being used to accomplish the grant goals and 3) the importance of genomics, pharmacogenetics and biospecimen collection and storage in making targeted treatments available to patients. The first presentation by George Sledge, MD included an overview of the IU/DOD grant, the "omics" involved in the research, the desired outcomes and how advocates can support this research. The second presentations were offered by Jenny Chang, MD giving an overview of genomics in cancer and David Flockhart, MD, PhD providing an overview of pharmacogenetics. The third presentation by Ann Thor, MD discussed the operational issues in collecting and storing biospecimens, especially in multi-centered, multi-country research. The sessions were archived for later playback for those that are unable to attend the live session.

Accomplishments

- Patient advocates participated in monthly core conference calls and meetings with Center staff.

- Web site content was developed to provide information about the focus of the Center, the science fundamental to the work of the center and resources for patients and advocates.

- A one-page summary of *Predicting Response and Toxicity in Patients Receiving Chemotherapy for Breast Cancer: A Multicenter Genomic, Proteomic, and Pharmacogenomic Correlative Study* was developed.

- An Advocate Lecture Series was offered during the month of March to 60-70 advocates representing national, regional and local advocate organizations.

Conclusions

- Advocates and researcher can work together to help ensure the success of research studies.

- The model of the development of patient educational material by advocates with input from researchers and staff proved to be successful.

- Developing a network of advocates, advocate organizations, and researchers can increase awareness and understanding of specific research projects.